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Identification of metabolites of lobeline in the rat urine by liquid chromatography–tandem mass spectrometry

Wei Song, Zhihong Peng, Baoying Ge, Fengmei Han, Yong Chen *

Hubei Province Key Laboratory of Biotechnology of Chinese Traditional Medicine, Hubei University, 430062, China

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Abstract

This is a report about the analysis of lobeline and its metabolites in rat urine by using high-performance liquid chromatography–electrospray ionization ion trap tandem mass spectrometric method (LC/MS^n). The urine of healthy rat were sampled from 0 to 24 h after administered a single dose of lobeline (3 mg/kg) by oral gavage, then centrifuged at 10,000 rpm for 10 min to get the supernatants. The supernatants were purified by solid-phase extraction (SPE) with a C_{18} cartridge. After the above purified process, the purified urine were injected into a reversed-phase C_{18} column with mobile phase of methanol/water (70:30, v/v, adjusted to pH 3.5 with formic acid) and detected by an on-line MS^n system. The identification and structural elucidation of the metabolites were performed by comparing their changes in molecular mass (ΔM), full-scan MS^n spectra with those of the parent drug. Ten metabolites of lobeline were found in rat urine. All the metabolites were reported for the first time.
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Keywords: LC–MS^n; Lobeline; Metabolite; Rat

1. Introduction

Lobeline (Fig. 1) is a lipophilic, non-pyridino, alkaloidal constituent of *Lobelia inflate* LINN., also known as *Rapuntium inflatum* MILL., Indian weed, pukeweed, asthma weed, gagroot, vomitwort, bladderpod, eyebright, and Indian tobacco [1]. It has many nicotine-like effects, including tachycardia and hypertension [2], bradycardia and hypotension in anesthetized rats [3], hyperalgesia [4], as well as analgesia after intrathecal, but not after subcutaneous, administration [5], anxiolytic activity [6], and improvement of learning and memory [7]. Interesting, intrathecal administration of lobeline also inhibits the analgesic effect of epibatidine (a potent nAChR agonist) [8,9]. Lobeline was previously investigated as a therapeutic agent to treat tobacco dependence and it has been demonstrated to inhibit the effect of amphetamines in behavioral and neurochemical assays [10]. A recent study indicated that lobeline has both temperature-dependent and temperature-independent neuroprotective effects against METH toxicity [11,12]. Compared with the comprehensive investigations for therapeutical purpose, the study on its metabolism is limited. To our knowledge, there was no paper have been reported about the metabolites of lobeline yet.

LC–MS^n technique which was considered less time consuming and less labor intensive was used to analyze drug metabolites in biological materials [13–16]. This approach has high sensitivity and specificity. Besides, it is considerably less time consuming and less labor intensive than other methods, such as HPLC and GC–MS. In addition, MS/MS technique has made it possible to acquire rich structural informative data from pseudomolecular ions of analytes of interest. The identification and structural elucidation of drug metabolites using LC–MS^n method are based on the similarity of structural feature between the parent drug and its metabolites. The MS^n product ion spectrum of each metabolite can provide detailed substructural information. So, using the product ion mass spectrum of the parent drug as a substructural template, the structure of metabolites may be rapidly characterized even without standards of metabolite [13,17–21].

* Corresponding author. Tel.: +86 27 88663590; fax: +86 27 88663590.
E-mail address: cy101610@npc.gov.cn (Y. Chen).

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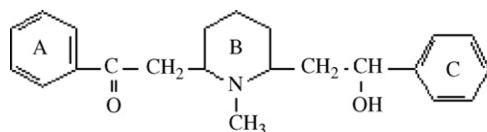

Fig. 1. Structure of lobeline.

The aim of this work was to develop a sensitive and specific LC/MS^n method to identify metabolites of lobeline and elucidate its structure in biological fluids.

2. Materials and methods

2.1. Chemicals and reagents

Lobeline was purchased from TianJin YiFang Co., Ltd. (TianJin, China). Methanol was of HPLC grade (Fisher Chemical Co., Inc., CA, USA). Water was deionized and double distilled. All other reagents were of analytical reagent grade. Stock solution of lobeline was prepared by dissolving lobeline in methanol to the concentration of 1.0 mg/mL.

2.2. Apparatus

LC/MS (MS^n) experiments were performed on an LCQ Duo quadrupole ion trap mass spectrometer (Thermo-Finnigan, Corp., San Jose, USA) with Agilent 1100 Series G1311A Quat pump and G1313A autosampler using positive/negative electrospray as ionization process. The software Xcalibur version 1.2 (Finnigan) was applied for the system operation and data collection. The urine samples were purified using a C_{18} solid-phase extraction cartridge (3 ml/200 mg, AccuBond II, Agilent). A high-speed desk top centrifuge (TGL-16C, Shanghai Anting Scientific Instrument Factory, Shanghai, China) was used to centrifuge urine samples.

2.3. Chromatographic conditions

The separation of lobeline and its metabolites was performed using a reversed-phase column (Zorbax Extend-C18, 3.0 mm × 100 mm, 3.5 μm, Agilent, USA) connected with a guard column (cartridge 2.1 mm × 12.5 mm, 5 μm, Agilent) packed with the same packing material, and the analytes were detected by an on-line LC/MS^n detector. The temperature of the column was set at 25 °C. The mobile phase was consisted of methanol and 2.0 mmol/L ammonium acetate (70:30, v/v, adjusted to pH 3.5 with formic acid). The flow rate was 0.2 mL/min. The injection volume of the purified urine was 20 μL.

2.4. Mass spectrometric conditions

Mass spectrometry was carried out on an LCQ Duo quadrupole ion trap mass spectrometer (Thermo-Finnigan, Corp., San Jose, USA). The identification and structrural elucidation of lobeline and its metabolites were performed in electrospray positive ion mode, while the phase II metalolites of lobeline were also detected in electrospray negative ion mode to validate its structure. Nitrogen was used as the sheath gas (80 arbitrary units). The MS analysis were performed under automatic gain control conditions, using a typical source spray voltage of −60 kV, a capillary voltage of −37 V and a heated capillary temperature of 250 °C. The other parameters, including the voltages of octapole offset and tube lens offset, were also optimized to achieve the maximum abundance of the ions of interest by the automatic tuning procedure. The MS^n product ion spectra were produced by collision induced dissociation (CID) of their molecular ion $[M + H]^+$ in selected reaction monitoring (SRM) mode. The energy of CID was 38% arbitrary units. Data acquisition was performed in full-scan LC/MS and LC/MS^n.

2.5. Samples preparation

2.5.1. Collection of rat urine samples

Six Wistar rats (180 ± 5 g, Hubei Experimental Animal Research Center, China) were housed in metabolic cages for the collection of urine. The rats were fasted for 24 h but with access to water, then administered a single dose of lobeline (3 mg/kg weight) by oral gavage. The rat urine were sampled up to 24 h after lobeline administration and centrifuged at 10,000 rpm for 10 min at room temperature, and the supernatants were collected and stored at −20 °C until further purification.

2.5.2. Purification of urine sample

Solid-phase extraction (SPE) with a C_{18} cartridge was used to purify the above supernatants of the urine samples. Before the cartridge was used, the cartridge was conditioned with 1 mL of MeOH followed by 1 mL of water, then the sample was passed through the cartridge and washed with 2 mL of water to remove the impurity. One mililiter of methanol was added to elute the analytes. The methanol effluent was centrifugated at 10,000 rpm for 10 min at room temperature, the supernatants were stored at −20 °C until for LC/MS^n analysis.

3. Results and discussion

3.1. LC–MS and LC–MS^n analysis of lobeline

The LC/MS and LC/MS^2 analysis of lobeline were performed in electrospray positive ion mode. Lobeline was eluted at 2.31 min under the experimental conditions (Fig. 2A). The full-scan mass spectrum of lobeline gave the protonated molecular ion $[M + H]^+$ at m/z 338. The MS^2 spectrum of the molecular ion at m/z 338 contains seven main product ions at m/z 320, 218, 216, 200, 105, 98 and 96 (Fig. 2B). The fragment ion at m/z 216 can lead to a MS^3 product ion at m/z 96 (Fig. 2C), and fragment ion at m/z 218 can lead to a MS^3 product ion at m/z 98 (Fig. 2D). The proposed fragmentation of lobeline was shown in Fig. 3. The product ions and the corresponding neutral fragment loss were the characteristic structural information of lobeline, and were the sound basis to identify metabolites of lobeline.

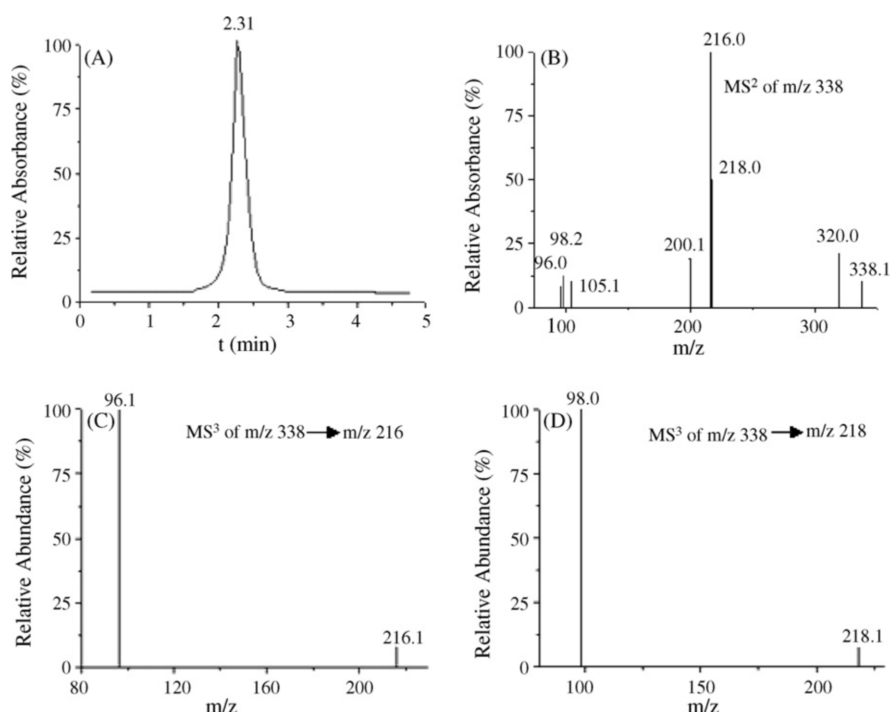

Fig. 2. (A) LC/MS^2 chromatogram of lobeline; (B) MS/MS product ion spectrum of lobeline; (C) from *m/z* 338 to *m/z* 216 to *m/z* 96; (D) MS^3 of *m/z* 338 on *m/z* 218.

3.2. LC–MS and LC–MS^n analysis of metabolites

Possible metabolite structures were considered based on the structure of lobeline and known common metabolic pathways. The full-scan mass spectra of rat urine samples before and after lobeline administration were compared to search the possible metabolites. Lobeline and its 10 metabolites with their protonated molecular ions $[M+H]^+$ at *m/z* 340, 354, 370, 356, 372 (two isomers), 514, 516, 530 and 532 were detected in rat urine after lobeline (3 mg/kg weight) administration. Then, the metabolites were analyzed by LC/MS^n in SRM mode to obtain their MS^n spectra (shown in Fig. 4).

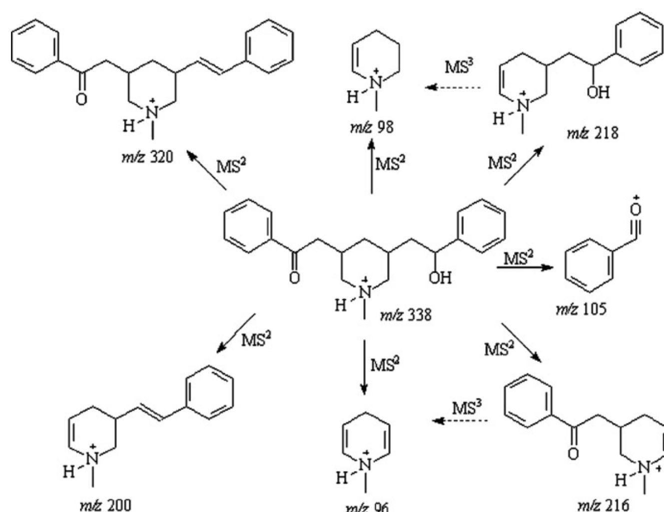

Fig. 3. The proposed fragmentation pathway of lobeline.

The molecular weight and MS fragmentation characteristic of each metabolite were compared with those of lobeline for more precise structural elucidation of metabolite. Among them, the retention time, the MS^2 and MS^3 spectra of the molecular ion at *m/z* 338 (M0) (Fig. 4A and B) were almost the same as those of lobeline. Therefore, M0 can be affirmed as the unchanged lobeline. The structure of metabolites were interpreted as follows.

The molecular ion of M1 (*m/z* 340) and its main MS^2 product ions at *m/z* 322 and 218 (Fig. 4C) were all 2 Da more than the molecular ion of M0 and its main MS^2 product ions at *m/z* 320 and 216. The product ion of M1 at *m/z* 218 can lead to a characteristic MS^3 product ion at *m/z* 98 (Fig. 4D). Furthermore, the characteristic MS^2 product ions of M0 at *m/z* 200 and 98 were also present in the MS^2 spectrum of M1, whereas the characteristic MS^2 product ions of M0 at *m/z* 216 and 96 disappear from the MS^2 spectrum of M1. So, M1 can be confirmed as the reduction product of lobeline, and the di-hydrogenating position was the unique carbonyl of lobeline.

The molecular ion of M2 (*m/z* 354) and its main MS^2 product ions at *m/z* 336 and 234 (Fig. 4E) were all 16 Da more than the molecular ion of M0 and its main MS^2 product ions at *m/z* 320 and 218. Furthermore, the characteristic fragment ions of M0 at *m/z* 216 and 105 were also present in the MS^2 spectrum of M2, and the fragment ion of M2 at *m/z* 216 can lead to a MS^3 product ion at *m/z* 96 (Fig. 4F). These results indicated that M2 was the hydroxylating product of M0, and the hydroxylating position was located at C ring.

The molecular ion of M3 (*m/z* 370) and its main MS^2 ions at *m/z* 352, 250 and 232 (Fig. 4I) were all 32 Da more than the molecular ion of M0 and its main MS^2 ions at *m/z* 320, 218

and 200. The MS^2 fragment ion of M3 at *m/z* 216 can lead to a MS^3 product ion at *m/z* 96 (Fig. 4J). Thus, M3 was the dihydroxylating product of M0, and the hydroxylating position was located at C ring.

The molecular ion M4 (*m/z* 356) and its main MS^2 ions at *m/z* 338 and 234 were all 16 Da more than the molecular ion of M1 and its main product ions at *m/z* 322 and 218. The characteristic product ions of M1 at *m/z* 218 and 200 were also present in the MS^2 spectrum of M4 (Fig. 4G). The MS^2 fragment ion of M4 at *m/z* 218 can lead to a MS^3 product ion at *m/z* 98 (Fig. 4H). The results indicated that M4 was the hydroxylating product of M1, and the hydroxylated position was located at either A ring or C ring.

The molecular ion of M5 and M6 (*m/z* 372) can lead to five main MS^2 ions at *m/z* 354, 250, 234, 218 and 200 (Fig. 4K), and the fragment ion at *m/z* 218 can lead to a MS^3 product

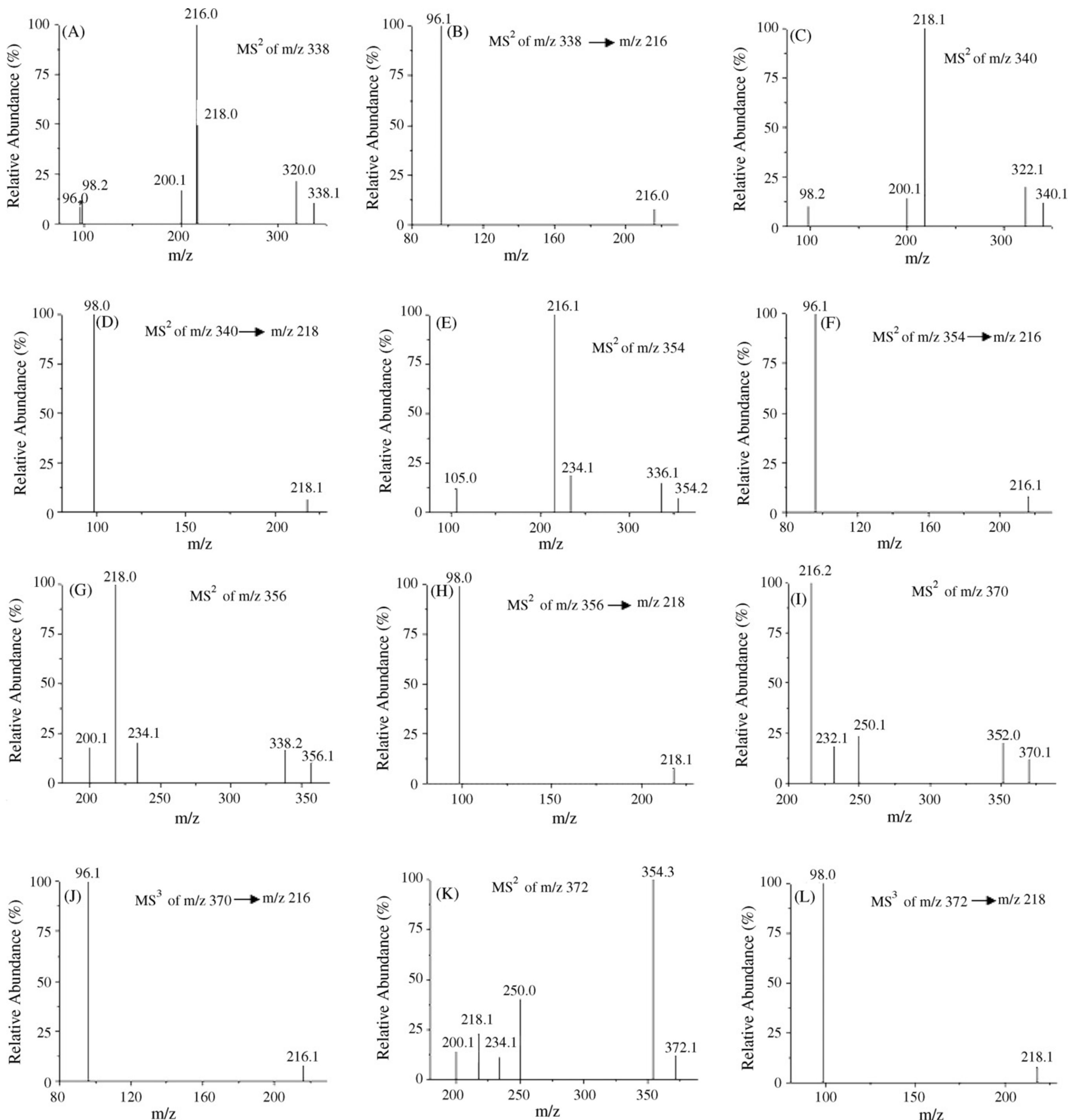

Fig. 4. LC/MS^2 chromatograms and MS^n spectra of the metabolites of lobeline.

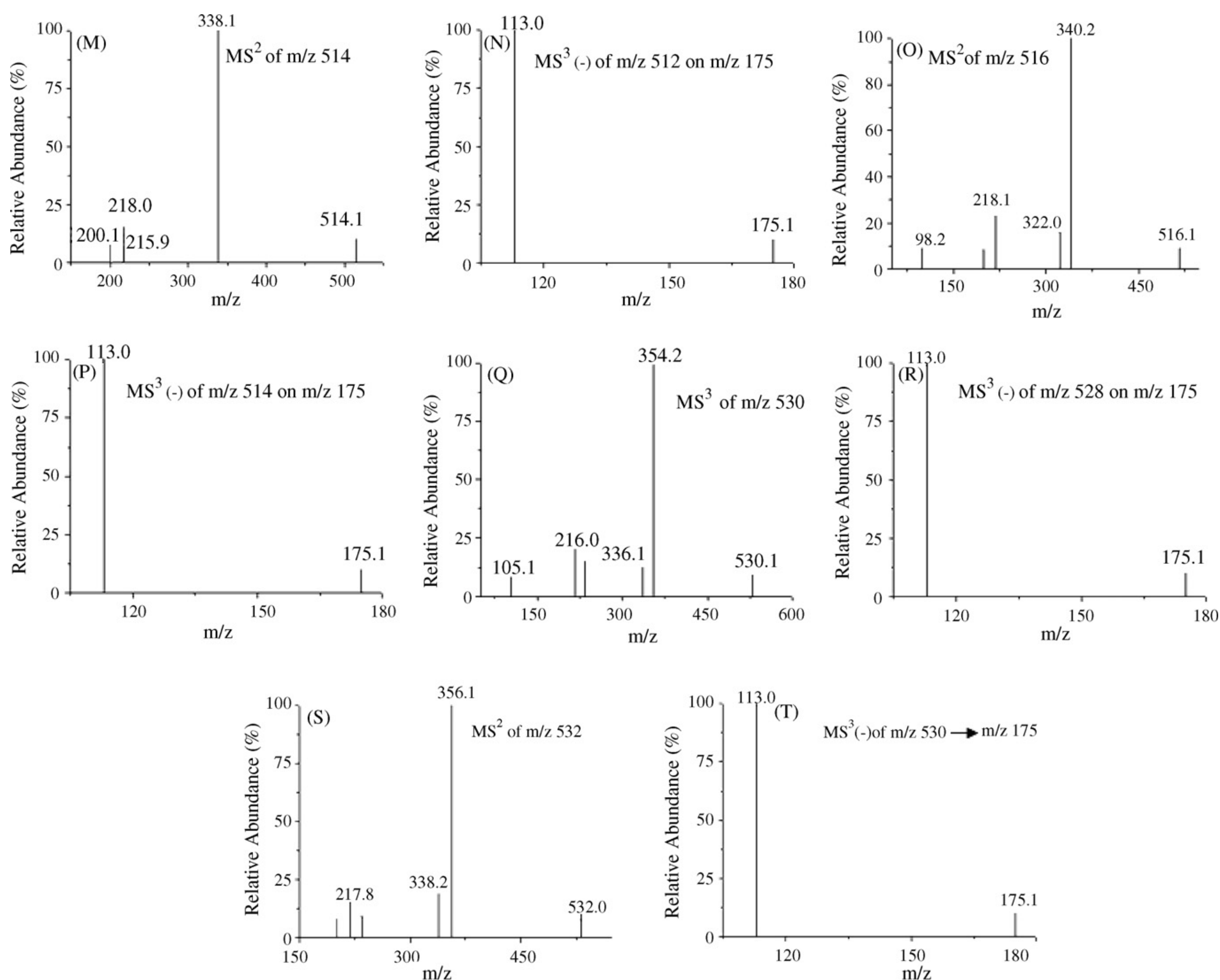

Fig. 4. (*Continued*).

ion it *m/z* 98 (Fig. 4L). The molecular ion of M6 and its fragment ions at *m/z* 354 and 250 were all 32 Da more than the molecular ion of M1 and its main product ions at *m/z* 322 and 218. Thus M6 could be confirmed as the di-hydroxylated product of M1. The MS^2 fragment ions of M6 at *m/z* 218 and 200 shown that M6 is a di-hydroxylating product of M1 with the bis-hydroxylation on either A ring or C ring, whereas the MS^2 fragment ions of M5 at *m/z* 250 and 234 indicated that M5 was a di-hydroxylating product of M1 with one hydroxylation on B ring and an other hydroxylation on either A ring or C ring. So, M5 and M6 may be a mixture of two isomers.

The molecular ions of M7 (*m/z* 514), M8 (*m/z* 516), M9 (*m/z* 530) and M10 (*m/z* 532) lose a neutral fragment 176 Da (Gla) to produce the most abundant daughter ions at *m/z* 338 (Fig. 4M), 340 (Fig. 4O), 354 (Fig. 4Q) and 356 (Fig. 4S), respectively. The MS^3 spectra of *m/z* 514 → 338, 516 → 340, 530 → 354 and 532 → 356 were almost the same as the MS^2 spectra of the molecular ions of M0, M1, M2 and M4, respectively. In addition, the ESI negative ion full-scan LC/MS spectrum of rat urine after drug administration yield their corresponding de-protonated molecular ions $[M-H]^-$ at *m/z* 512 (M7), 514 (M8), 528 (M9), 530 (M10), and all of the de-protonated molecular ions gave a MS^2 fragment ion at *m/z* 175 and subsequently MS^3 fragment ion at *m/z* 113 in ESI negative ion mode (Fig. 4N, P, R, T). This is the characteristic cleavage fragment of glucuronide conjugates [22,23]. Thus, M7, M8, M9 and M10 can be confirmed as the glucuronide conjugate of M0, M1, M2 and M4, respectively.

The results also indicated that the parent drug and its metabolites can be detected for up to 24 h in rat urine sample after lobeline (3 mg/kg weight) administration. Regardless of the MS response difference of the metabolites, the relative contents of the metabolites, calculated by their LC/MS^2 chromatographic peak area, were as follows: M2 > M4 > M0 > M1 > M7 > M9 > M10 > M3 > M6 > M5 > M8. Because the polarity of metabolites were enhanced, so they were eluted ahead of the parent drug. Based on the structure of the metabolites, the proposed major metabolic pathway of lobeline in rats was shown in Fig. 5.

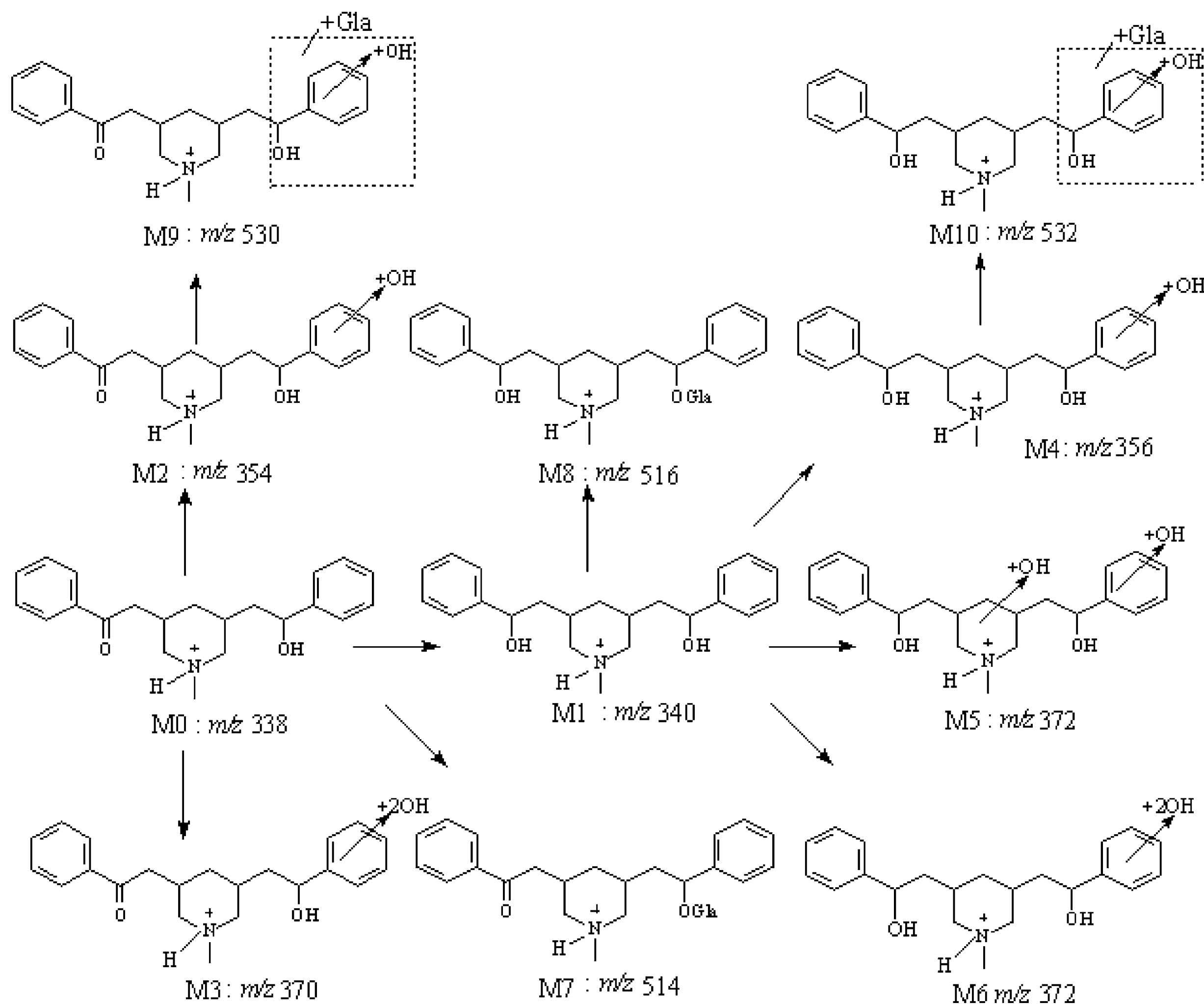

Fig. 5. Proposed metabolic pathway of lobeline in rat *in vivo* (Gla = glucuronic acid).

4. Conclusions

LC–MS^n ion trap with electrospray ionization method was very suitable for the identification of lobeline and its metabolites in rat urine. Ten metabolites, included six phase I metabolites and four phase II metabolites, were found in rat urine for the first time.

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